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Extraction, Purification, Assay, and Antimicrobial activity of Bromelain from Pineapple

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Abstract
Pineapple- Ananas comosus (Morris) is a fruit of the tropics. Bromelain, an enzyme, is extracted from the stem, fruit, skin, pulp, and leaf. The crude enzyme having proteolytic activity was purified and precipitated. Protein estimation and its assay were done by Lowry's method & GDU respectively. The molecular weight was determined by SDS-PAGE. Bactericidal activity was observed against both Gram-positive bacteria-Streptococcus and Gram-negative Bacteria-Salmonella by well method and tube method.

Keywords: *Ananas comosus, Lowry's method, SDS-PAGE, bactericidal, Streptococci, Gram + bacteria, Salmonella, Gram- bacteria*

1. Introduction
Bromelain is a class of sulfhydryl-containing proteolytic enzymes found in varying amounts throughout the Morris- Pine apple's plant body. Bromelain was first used to refer to any protease derived from plants in the Bromeliaceae family. Bromeliaceae is a plant family that produces a large number of proteases that appear to play no role in plant growth and development [1]. However, it is well-known for its clinical applications, which include tumor growth prevention, improved blood coagulation, antibacterial action, and anti-inflammatory properties. Pineapple [Morris], a member of the Bromeliaceae family, is a rich source of carbohydrates, sugar, organic acid, vitamins, crude fiber, and minerals, and is one of the most significant tropical fruit crops, after bananas and citrus fruits. Bromelain is derived from pineapples ('Ananas comosus'), which are native to Central and South America and have traditionally been used to treat a range of ailments. Bromelain was initially isolated from pineapples in 1957, and it quickly gained popularity as a possible medicinal ingredient. The enzyme bromelain is a natural detoxifier that is good for the digestive system and helps with weight loss and nutrition. It is also well-known for its anti-inflammatory and anti-clotting properties. The most popular and produced commercially Pineapples are the King and Queen. The King variety plant is a short 1–1.5meter herbaceous perennial with 30 cm or more trough-shaped leaves 30–100 cm long, all surrounded by a stout stem. It's a late-maturing pineapple variety that's India's most popular commercial variety [2]. Fruit is large, rectangular in shape, and slightly tapering towards the crown, weighing 2-3kg. In Australia, Queen is also known as 'Common Rough.' Because of its high sugar content and unsuitability for canning, this group of cultivars is widely dispersed and widely farmed for fresh fruits over the world. The plant is small, with dark purplish-green leaves that are short and highly spiky, compact, cold, and disease

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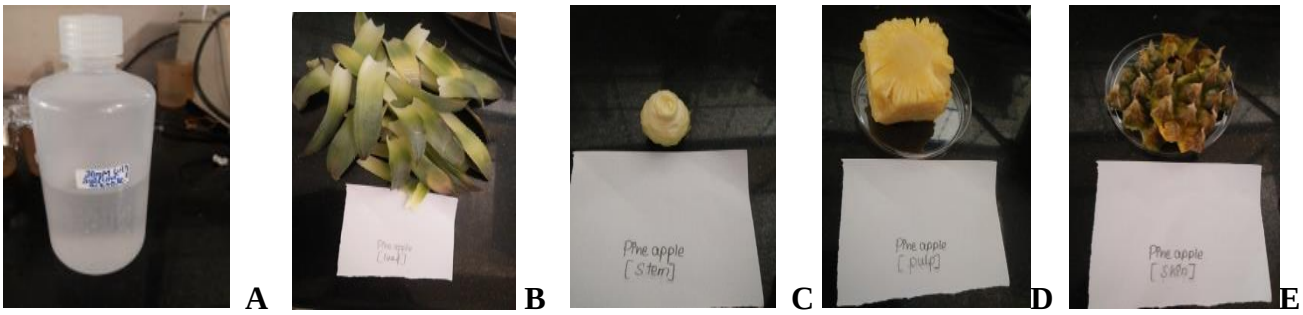
resistant. Ananas morris provides 70% of Malaysia's fresh pineapple fruit. It is the most popular variety in South Africa, Queensland, and the Philippines. The plant is compact and cold and disease resistant with dark purplish-green leaves that are short and spiky. The fruit is conical, dark yellow, and has deep eyes, and it requires a thick cut to remove the peel completely; it is less fibrous, small, with a delicate core that is juicy and sweet and has a great flavor. The fruits are spiky and golden yellow when fully ripe, having a pleasant aroma and flavor. The fruits are harvested when the eyes turn yellow [3].

2. Materials and Methods

Pineapples, blender, sterile distilled water, dialysis, funnel, beaker, filter paper, buffer, knife, measuring cylinder, Petri plates, Luria Bertani Agar, magnetic stirrer, conical flask, alcohol, corkborer, spreader, Incubator, Autoclave, LAF, Water bath, Sodium Chloride, Sodium Hydroxide, Ammonium acetate, Muller Hinton Agar, SDS PAGE (Refer Figure 1 & 2).

2.1. Extraction

Fresh pineapple stems were taken, peeled off, chopped into small pieces, and weighed after being rinsed with 0.1 percent hydrogen peroxide solutions. The mass of the weighed mass was determined to be 1,500 grams. The juice was extracted from the fresh pineapples stem section using a homogenizer in the presence of a sodium acetate buffer solution and then filtered. A total of 500 mL of filtrate was collected. At a quantity of 1 mg. per kg of the stem, benzoic acid was applied as a preservative. The resulting filtrate was dubbed crude extract and was employed as a source of stem bromelain. Fully ripe pineapple fruits were selected, washed, and cut into thin slices. The mass weighed in at 600 grams. A homogenizer was used to extract the juice, filtered, and collected in a beaker The filtered bulk was approximately 300 ml, with 0.6-gram sodium benzoate added and was labeled as cruite extract.



A B C D E

Figure 1 Crude extraction: A-Buffer B-Leaves C-Stem D-Pulp E- Skin

Figure 2 Crude Extracts of yhe Samples

2.2. Ammonium Salt Precipitation

Salting out is a technique used to purify proteins by altering their solubility (Figure 3). Two distinct effects were observed. The solubility of the portein increases with the increasing salt concentration (Increasing the ionic strength) and is termed as salting in. As the salt concentration is increased further, the

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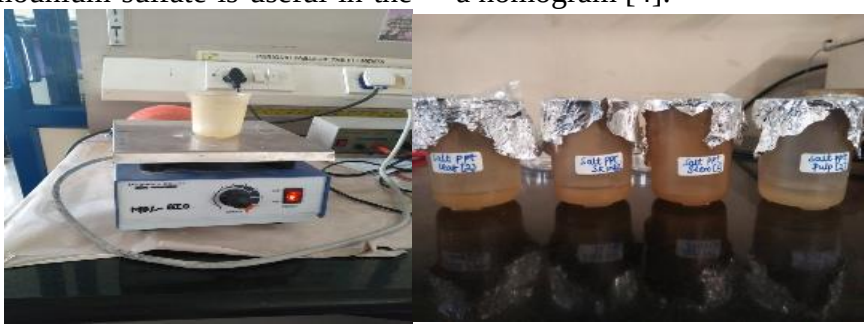
solubility of the protein begins to decrease. Since the proteins have varying solubility, the salting-out technique using ammounium sulfate is useful in the purification of the protein. The amount of the ammonium sulphate to be used was determined from a nomogram [4].



A B

Figure 3 A. Ammonium Salt Precipitation Using A Stirrer. B. Ammonium Sulphate Extraction

2.3. Substrate Selection

Bromelain is measured as GDU (Gelatin digestion unit) by assessing its digesting activity on gelatin. Bromelain's activity is roughly similar to 1,200 GDU per g. For the study of bromelain activity, gelatin was chosen as the substrate. Gelatin digestion unit analytical technique for crude stem bromelain assay he rates of degradation of gelatin (substrate) was evaluated using crude stem bromelain extract. Various reagents were prepared, including gelatin (5%) (reagent A) as a substrate, hydrogen peroxide solution (3%) (reagent B), formaldehyde solution (37%), 0.05N sodium hydroxide (reagent C), 100 mM sodium acetate buffer with 2.6 M sodium chloride (reagent D), and 0.05N sodium hydroxide (reagent E). In a beaker labeled "test solution," one mL of crude stem extract was placed. With 0.05 N NaOH, and the pH at 6.0. Reagent A (25 mL) was added and heated in a water bath at 45°C. 0.1 ml of reagent B was added and stirred after 20 minutes of incubation at 45°C. The pH of the beaker was adjusted to 6.0 with 0.05N NaOH after it was taken from the water bath. With continuous stirring, 10ml of reagent C was added. With 0.1 N NaOH, titration was done to pH 9.0. The test solution's titration volume was recorded (Table 1).

$$= \frac{\text{(Volume of test-volume of blank) (N) (14) x 1000}}{\text{Mg enzyme/RM}}$$

N=Normality of NaOH 14=mg N per millimole N, mg enzyme= amount of concentration of the enzyme bromelain present in 1ml of crude extract, RM= reaction mix. The concentratin of the enzyme was found to be 0.46mg/ml. The test solution and the blank solution were both run simultaneously. To begin, 25 mL of reagent A and 0.1 mL of reagent B were added to the beaker placed in the water bath at 45°C. After 20 minutes, 1.0 ml of bromelain solution was added, mixed, and incubated for another 5 minutes [5].

Table 1 The volume of NaoH in µl

Test tube	Reagent A	Sample in µl	Incubate at 45'c for 20m in a water bath	Reagent B	pH adjusts blank of both Test tube 6.9 a-(HclBase (NaoH)	Formaldehyde was Add 1ml	Titration against NaoH Adjust To pH	The volume of NaoH in µl
Blank	2.5	100 µl		10 µl				406
Leaf Bromelain	2.5	100 µl		10 µl				460
Pulp Bromelain	2.5	100 µl		10 µl				435

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Skin Bromelain	2.5	100 µl		10 µl				480

2.4. Estimation & Purification

The crude extracts of stem and fruit bromelain were centrifuged for 10 minutes at 4^0 C at 6,000 rotations per minute. The supernatant was collected, and the enzyme assay was carried out (Table 2). Finally, ammonium sulphate precipitation and dialysis were used to purify the samples.

2.5. Quantitative Estimation of Stem and Fruit Bromelain by Lowry's Method

Lowry's method was used to evaluate the concentration of proteins (bromelain) in the stem and fruit, with slight variations [6]. In the test tube, different dilutions of BSA solution were prepared using BSA solution (100 g/ml) and distilled water as a standard to set to a final volume of 1 ml. BSA concentrations ranged from 0.02 to 0.1 mg/ml. The 0.1 ml of crude stem and fruit bromelain enzyme, were placed in test tubes numbered 10, 11, and 12, and the final volume was built up to 1 ml with distilled water. These were tubes containing samples of unknown concentrations (Figure 4). To each of the tubes, the addition of 5 ml of alkaline copper sulphate reagent did and was thoroughly mixed. Incubated at room temperature for 10 mins. To each of these tubes, 0.5 ml of FC (Folin Cocalteau) reagent was added and incubated in the dark for 30 min. All of the samples were analyzed with a spectrophotometer at 660 nm. A standard calibration curve from BSA was obtained, the absorbance was plotted against the protein content. The concentration of the unknown samples was obtained from the graph (Table 2).

Table 2 Quantitative Estimation of Stem and Fruit Bromelain by Lowry's Method

Sl.No.	Amount of std BSA(µl)	Amount of Distilled water (µl)	The volume of a complex-forming reagent	Incubate at room temperature for 30min	Folins reagent	Incubate at room temperature for 10min	OD at 660nm
1	Blank 1	200	3ml		0.3ml		0
2	Blank 2	200			0.3ml		0
3	10	190			0.3ml		0.069
4	20	180			0.3ml		0.13
5	40	160			0.3ml		0.24
6	80	120			0.3ml		0.4
7	120	80			0.3ml		0.60
8	160	40			0.3ml		0.65
9	200	0			0.3ml		0.88
10	Leaf 10(µl)	190			0.3ml		0.10
11	Pulp 10(µl)	190			0.3ml		0.06
12	Skin 10(µl)	190			0.3ml		0.10

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Figure 4 Estimation of Proteins by Lowry's Method

2.6. Salting Out

Ammonium sulphate precipitation for stem bromelain was accomplished by adding 6.6 gm of ammonium sulphate salt, pinch by pinch, to 15 ml supernatant obtained after centrifugation under ice-cold conditions for 45 minutes with constant stirring on a magnetic stirrer. Similar activity was carried out for the same period with fruit bromelain. Bromelain sample solutions from stems and fruits were incubated overnight at 4°C. After incubation, the precipitated enzymes were centrifuged at 10,000 rpm for 10 minutes at 4°C. The pellet was recovered and dissolved in 10 mL of 10 mM Tris HCl buffer before being dialyzed.

Figure 5 Ammonium Sulphate Saturation

2.7. Dialysis

The above-mentioned solution was placed in a dialysis bag and examined for sample leakage. The dialysis bag was then suspended in a 100 mM phosphate buffer-NaCl solution in a beaker. This setup was kept in the refrigerator overnight to keep it cool. This procedure was followed for both the stem and fruit bromelain. (Refer Figure 5,6 & 7)

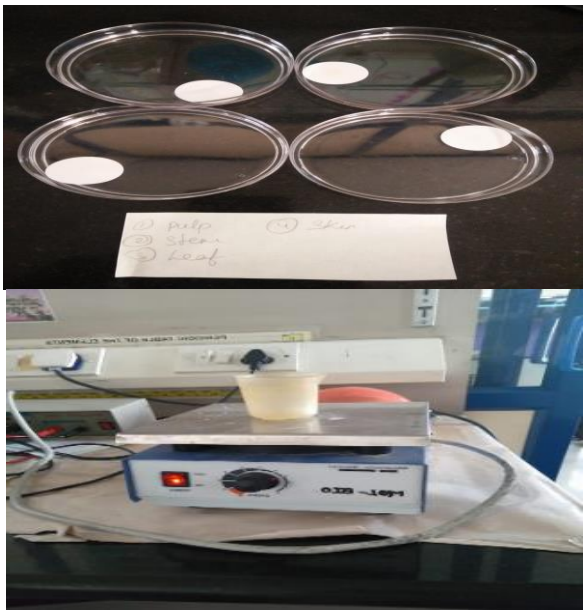

Figure 6 Dialysis

2.8. Bag Dialysis

Fractionation of Bromelain juice was done using ammonium sulphate, dialysis membrane. The dialysis membrane was pre-cooled after being placed beaker with sodium citrate buffer. A magnetic stirrer has been used for stirring the contents in a cold condition. 6 dialysis membrane strips, each 9 cm

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long, were taken (one for each sample). They were boiled for 10 minutes in distilled water at 80°C, then 10 minutes in a 2% sodium bicarbonate solution, water, rinsed with distilled water for the pores to open. To prevent leakage, one end of the dialysis membrane was shut. Equal amounts of crude enzyme extracts (2 mL) were pipetted into separate dialysis bags, with the other end sealed as well. The dialysis bags were immersed in buffer solution and placed overnight on a magnetic stirrer.

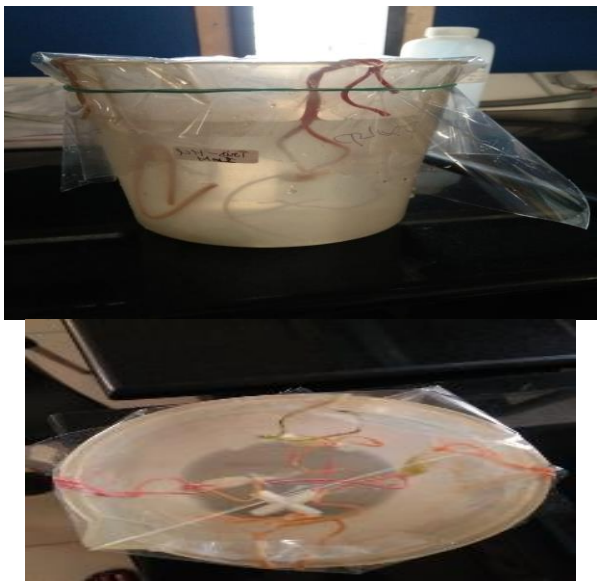

Figure 7 Dialysis Dipped with Tris-HCl

Different extracted enzymes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (bromelain). The enzymes were concentrated and put onto a 3.5 percent stacking gel, then electrophoresed at 200 V on a 12 percent separating gel (BioRad, Hercules, California, USA) until the Coomassie dye-stained protein band reached the gel bottom. Isolated bromelain was photographed on SDS PAGE.

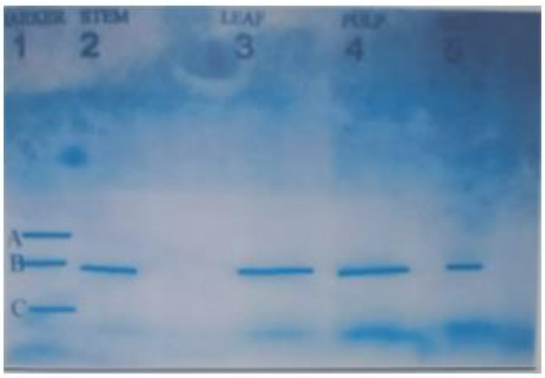

Figure 8 SDS Page

3. Antimicrobial Activity

Table 3 LB Media Composition

Components	Quantity
Tryptone	10g
yeast	5g
Sodium chloride	10g
Distilled water	1000ml

Pure cultures of *Salmonella* NCTC 7297 and *Streptococcus equi* were inoculated into four test tubes containing 5ml each of sterile LB broth. The tubes were incubated at 37^0C for 24 hrs. Turbidity of the broth indicated growth (Refer Table 3).

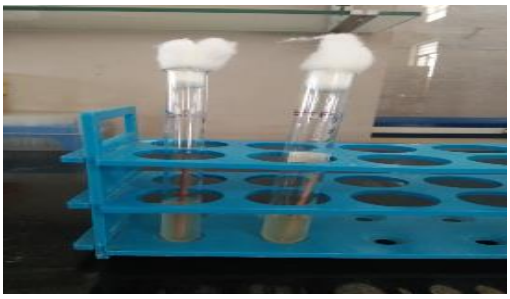

Figure 9 Growth of Salmonella & Streptococci on LB Broth

To the sterile LB agar plates, wells were made using a corkborer of 2mm. The culture of *Salmonella* and *Streptococci* were inoculated by the spread plate technique. 10µl of the crude bromelain extract from different plant parts was dispensed into each of the wells. The plates were incubated at 37^0 C, observed

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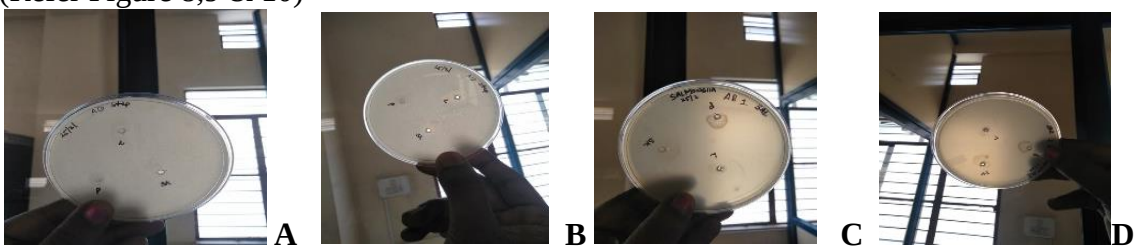
at 24hours for the zone of inhibition which was measured. (Refer Figure 8,9 & 10)



A B C D

Figure 10 Inhibition Zone Observed On the Developed Plates

Results & Discussion

Bromelain refers to the group of sulfhydryl-containing proteolytic enzymes derived from the *Ananas* fruit. Bromelain is mostly made up of a sulfhydryl proteolytic fraction. Peroxides, acid phosphatase, numerous protease inhibitors, and organically bound calcium are also present. It's a cysteine endopeptidase that breaks peptide bonds at the carbonyl group, such as arginine or aromatic amino acids like phenylalanine or tyrosine. A comparison of stem bromelain and fruit bromelain was carried out in this study. The juice extracted was referred to as a crude enzyme extract, and the activity of these crude extracts was measured, calculated using gelatin hydrolysis, and represented in the form of gelatin digestion units (GDUs) (Table 4).

Table 4 Results

Volume of sample	Protein conc(mg/ml)	Total protein (mg)	Activity(units/µl)	Units /ml	Total activity (unit)	Specific activity unit /mg
L-11	1	11	16.43	328.6	3,614.6	328.6
P-19	0.6	11.4	14.71	294.2	5,589.8	490.3
Sk-11	1	11	22.52	450.4	495,440	495440
L-11	0.9	9.9	38.55	771	8481	856.6
P-19	1.1	20.9	241.65	4833	91827	4393.6
Sk-11	1.4	15.4	20.43	408.6	4494	3210

The enzymatic activity of stem bromelain differs significantly from that of fruit bromelain, according to previous results on structural and kinetic investigations. Based on the findings, it was determined that stem bromelain had higher enzymatic activity than fruit bromelain. The crude fraction of stem bromelain obtained from stem, centrifuging at 6,000 rpm was observed. The enzyme was then precipitated out of the crude extract using ammonium sulphate precipitation. To remove the salt and other ions bound to the enzyme, the pellet was dissolved in 10 mM Tris HCl buffer, and dialysis was performed. The integrity and purity of bromelain protein were deduced from a single SDS-PAGE band of ion exchange extracted bromelain protein. The molecular weight of bromelain was approximately 30 kDa, according to the results of polyacrylamide gel electrophoresis of isolated bromelain components. This study found that purifying bromelain from the stem and fruit of the pineapple is structurally stable

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and has higher proteolytic activity than the crude extract and centrifugal fraction. According to the findings, stem bromelain has more GDU activity than fruit bromelain. The centrifugal fraction had higher enzymatic activity than the crude extract, indicating that this process needs to be improved to produce bromelain with higher proteolytic activity. Antimicrobial activity was noticed in Gram-positive bacteria than in Gram-negative bacteria. Furthermore, efforts must be made to develop a simple, cost-efficient, and successful technique for producing ultrapure bromelain with widespread applications.

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